

## VP35 Knockdown Inhibits Ebola Virus Amplification and Protects against Lethal Infection in Mice

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**Phosphorodiamidate morpholino oligomers (PMO) are a class of uncharged single-stranded DNA analogs modified such that each subunit includes a phosphorodiamidate linkage and morpholine ring. PMO antisense agents have been reported to effectively interfere with the replication of several positive-strand RNA viruses in cell culture. The filoviruses, Marburg virus and Ebola virus (EBOV), are negative-strand RNA viruses that cause up to 90% lethality in human outbreaks. There is currently no commercially available vaccine or efficacious therapeutic for any filovirus. In this study, PMO conjugated to arginine-rich cell-penetrating peptide (P-PMO) and nonconjugated PMO were assayed for the ability to inhibit EBOV infection in cell culture and in a mouse model of lethal EBOV infection. A 22-mer P-PMO designed to base pair with the translation start site region of EBOV VP35 positive-sense RNA generated sequence-specific and time- and dose-dependent inhibition of EBOV amplification in cell culture. The same oligomer provided complete protection to mice when administered before or after an otherwise lethal infection of EBOV. A corresponding nonconjugated PMO, as well as nonconjugated truncated versions of 16 and 19 base residues, provided length-dependent protection to mice when administered prophylactically. Together, these data suggest that antisense PMO and P-PMO have the potential to control EBOV infection and are promising therapeutic candidates.**

The *Filoviridae* family consists of only two genera, Ebola virus (EBOV) and Marburg virus (MARV), and belongs to the order *Mononegavirales*. Filoviruses possess a nonsegmented, single-stranded RNA genome of negative polarity that contains seven genes. The nontranscribed genomic ends, termed “leader” (3′ end) and “trailer” (5′ end), harbor *cis*-acting signals important for viral transcription, replication, and encapsidation. Transcription and replication of the viral genome requires viral proteins (VP) VP30 and VP35, the nucleoprotein (NP), and viral polymerase L (27, 28). Along with its role in viral RNA synthesis, VP35 has also been shown to act as a type I interferon antagonist (2). As the only viral surface protein, the glycoprotein (GP) is involved in binding and entry of the virion into host cells. The matrix protein VP40 functions in virus assembly and budding, and it has been suggested that the minor matrix protein VP24 also plays a role in these processes (7, 14).

Most MARV and EBOV species cause a severe hemorrhagic fever associated with fatality rates up to 90% in humans and nonhuman primates (8, 32). Due to the high mortality rates and the lack of any approved effective human therapeutic or vaccine (5), filoviruses have been classified as biological

safety level 4 (BSL-4) agents. Many approaches have been employed in attempts to develop effective therapies for EBOV, including the administration of nucleoside analogues (i.e., ribavirin), immune globulins, type I interferons and other cytokines, anticoagulants, and therapeutic vaccines (10, 13, 19, 20, 24, 37, 39, 40). To date, the highest survival rate (33%) observed in EBOV-infected nonhuman primates was obtained by the administration of an anticoagulant molecule, recombinant inhibitor of factor VIIa/tissue factor (11). Collectively, previous work suggests that treatment of disease symptoms or processes may increase the likelihood of survival. However, an approach that directly inhibits EBOV replication as a way to more effectively treat filovirus infections seems highly desirable.

Phosphorodiamidate morpholino oligomers (PMO) are a class of single-stranded antisense agents that have the same purine and pyrimidine bases as those of DNA but possess a backbone modified to contain a phosphorodiamidate linkage and morpholine ring (36). The molecular character of the PMO backbone results in near-complete resistance to nucleases (18) yet allows maintenance of aqueous solubility. PMO function by Watson-Crick base pairing and are most frequently designed to target mRNAs by complementary base pairing with the translation start site region, thus forming a steric blockade (reviewed in reference 15). To enhance uptake into cells, PMO have been conjugated to cell-penetrating arginine-rich peptides (6, 26). Both nonconjugated PMO and peptide-PMO (P-PMO) have been shown to successfully inhibit replication of several viruses in cell culture, including *Vesivirus* (35), mouse hepatitis virus (31), severe acute respiratory syndrome coronavirus (30), and several flaviviruses (6, 21).

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TABLE 1. PMO compounds and target locations in EBOV RNA

Compound	Sequence name	Target	Target location <sup>a</sup>	Sequence <sup>d</sup>
P-PMO (PMO conjugated to R <sub>9</sub> F <sub>2</sub> peptide) <sup>c</sup>	Leader	Leader	1–22 (–)	CGG ACA CAC AAA AAG AAA GAA G
	tss-NP	NP tss <sup>b</sup>	45–67 (–)	CGA ATA ACT ATG AGG AAG ATT AA
	108	3' NTR NP <sup>c</sup>	108–130 (–)	GAA ATT GTT ACT GTA ATC ACA CC
	3136	VP35 gene	3136–3115 (+)	GTT GTC ATC TTG TTA GAC CAG C
	11588	L gene	11588–11567 (+)	GTA GCC ATT TAA TAT CAA GAG G
	18959	Trailer	18959–18938 (+)	TGG ACA CAC AAA AAA GAA GAA A
	Scramble	N/A <sup>f</sup>	N/A	AGT CTC GAC TTG CTA CCT CA
PMO (nonconjugated)	3136	VP35 gene	3136–3115 (+)	GTT GTC ATC TTG TTA GAC CAG C
	3136 16-mer	VP35 gene	3133–3118 (+)	GTC ATC TTG TTA GAC C
	3136 19-mer	VP35 gene	3133–3115 (+)	GTC ATC TTG TTA GAC CAG C
	Scramble	N/A	N/A	TGT GCT TAC TGT TAT ACT ACT C

<sup>a</sup> Indicates the oligomer target nucleotides in the EBOV genome (–) or antigenome (+). Nucleotide designations refer to GenBank accession number AF086833.

<sup>b</sup> Transcription start signal of NP.

<sup>c</sup> 3' nontranslated region of the EBOV NP gene.

<sup>d</sup> Sequences are shown in 5' to 3' direction.

<sup>e</sup> R<sub>9</sub>F<sub>2</sub> peptide is described in Materials and Methods.

<sup>f</sup> N/A, not applicable.

For this study, PMO and P-PMO were designed to target six sequences in the genomic or antigenomic RNA of the EBOV species Zaire. We sought to select the most efficacious of the oligomers, based on their *in vitro* activities, for further evaluation in a mouse model of EBOV infection. Compared to other sequences, a VP35-specific P-PMO was able to inhibit EBOV replication in cell culture and greatly increase the survival of EBOV-infected mice when administered either prophylactically or therapeutically. A corresponding nonconjugated PMO was also effective as a prophylactic in mice. These data suggest that PMO compounds can specifically interfere with EBOV replication *in vivo* and thus hold interest for further therapeutic development.

#### MATERIALS AND METHODS

**Cells and viruses.** Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin. Zaire EBOV strain Mayinga or MARV strain Musoke was used for infection of cells in tissue culture (33, 34). The filoviruses were propagated in Vero or Vero E6 cells and titrated by a 50% tissue culture infective dose (TCID<sub>50</sub>) assay or standard plaque assay (25). All experiments with filovirus-infected cells or mice were performed in the BSL-4 facilities of the Philipps-University Marburg or the United States Army Medical Research Institute of Infectious Diseases (USAMRIID). Mice were infected with ~1,000 PFU of a mouse-adapted strain of EBOV (4).

**Design and synthesis of PMO and P-PMO.** PMO compounds were designed as sequences complementary to Zaire EBOV (GenBank accession number AF086833) and named for their target nucleotides within the EBOV genome or antigenome (Table 1). All PMO were synthesized and purified by AVI BioPharma Inc. (Corvallis, OR), as previously described (36). A set of PMO were covalently conjugated at the 5' end to an arginine-rich peptide, NH<sub>2</sub>-RRRRRRRRFFC-CONH<sub>2</sub>, designated as R<sub>9</sub>F<sub>2</sub>. The conjugation, purification, and analysis of R<sub>9</sub>F<sub>2</sub>-PMO compounds were performed as previously described (26). In this report, peptide-conjugated PMO are referred to as P-PMO and were used in all of the cell culture experiments. Both P-PMO and nonconjugated PMO were used in the *in vitro* translation and animal experiments and are duly indicated. A "nonsense" sequence (named "scramble") was prepared as a PMO and as a P-PMO to serve as a control for off-target effects of the respective chemistries.

**P-PMO treatment and infection of cells in tissue culture.** Unless otherwise stated, 10<sup>5</sup> Vero cells were seeded in 12-well culture plates and treated the next day with medium containing a final concentration of 5.0 μM P-PMO for 3 hours prior to viral infection. P-PMO-containing medium was removed during infection with either MARV or EBOV (multiplicity of infection [MOI] of 5 TCID<sub>50</sub> per cell). After infection, the inoculum was removed and replaced with DMEM containing 2% FCS and P-PMO where stated. Cells were then incubated for 24

to 72 h, as indicated, without changing the medium. Modifications to this protocol are noted in the figure legends and Results.

**TCID<sub>50</sub> assay.** Vero cells were grown in 96-well plates to 30 to 40% confluence. The cells were then inoculated with 10-fold serial dilutions of supernatant from P-PMO-treated and infected cells. At 10 to 12 days postinfection (dpi), when the cytopathic effect (CPE) had stabilized to a constant rate, the dilutions showing CPE were evaluated by light microscopy. The TCID<sub>50</sub>/ml was calculated using the Spearman-Kärber method (17).

**MTT cytotoxicity assay.** Vero cells were grown in 96-well plates to 60 to 70% confluence in normal growth medium supplemented with 10% fetal bovine serum. The cells were then washed with phosphate-buffered saline (PBS) and incubated with serum-free medium containing water or PMO compounds at the indicated concentrations. After 24 h, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent was added, and the cells were assayed as previously described (21).

**RT-PCR.** Vero cells were seeded into 24-well culture plates at a concentration of 10<sup>4</sup> cells per well and allowed to adhere overnight. At various time points, as stated in the figure legends and Results, cells were treated with the indicated amounts of P-PMO in 2 ml serum-free DMEM. Cells were infected with EBOV at an MOI of 5 TCID<sub>50</sub> per cell. After 1 hour, the virus inoculum was removed and replaced with DMEM containing 2% FCS and P-PMO, where applicable. At 24 or 48 h postinfection (hpi), the cell supernatant was removed, and total RNA was isolated using the RNeasy mini kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. A tenth of the eluted RNA was used in a OneStep reverse transcription-PCR (RT-PCR) (QIAGEN) according to the manufacturer's instructions, with EBOV-specific primers to amplify nucleotides 3660 to 4778 of genomic EBOV RNA (VP35/VP40) (GenBank accession number AF086833). MARV viral RNA (GenBank accession number Z29337) was detected by amplifying nucleotides 5890 to 6521 (GP gene). The PCR was performed for 27 cycles and included amplification of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA as a control. Ten percent of the RT-PCR products was resolved on a 2% agarose gel and stained with ethidium bromide.

**Cell-free translation assay.** The nucleotide sequence corresponding to a segment of EBOV VP35 mRNA (–98 to +39 relative to the translation initiator AUG codon; genomic bases 3020 to 3157), immediately followed by the protein coding sequence for firefly luciferase (without its initiator AUG codon), was subcloned into the polylinker of plasmid pCiNeo (Promega, Madison, WI). *In vitro*-transcribed RNA was generated via the plasmid T7 RNA polymerase promoter using the T7 MEGAscript kit (Ambion, Austin, TX). *In vitro* translations using a rabbit reticulocyte system (Promega) were carried out by mixing different concentrations of PMO with *in vitro*-transcribed RNA present at a final concentration of 1 nM. Luciferase light emission was read on an FLx800 microplate luminometer (Bio-Tek Instruments, Inc., Winooski, VT). The relative light units produced by each reaction (*n* = 3 per data point) were normalized to the mean of the water control reactions and expressed as percent inhibition of luciferase translation. Fifty percent effective concentration (EC<sub>50</sub>) values were determined with GraphPad Prism (San Diego, CA) graphing software.

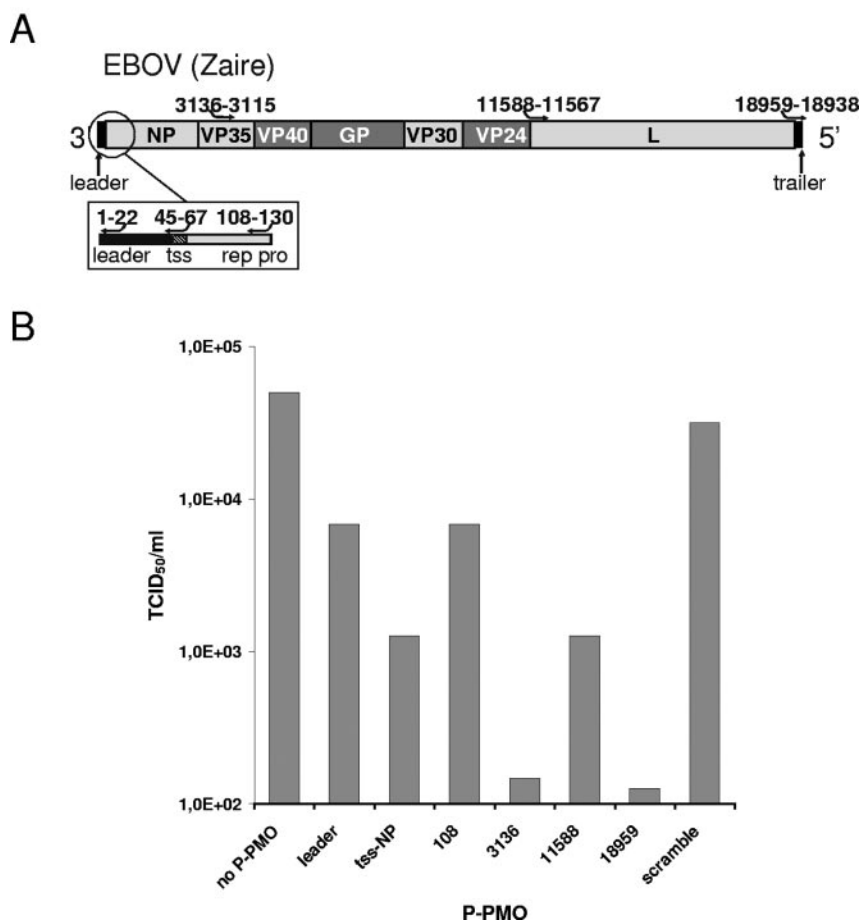


FIG. 1. Inhibition of viral replication by EBOV-specific P-PMO. (A) Schematic drawing of the EBOV Zaire genome and PMO target sequences. Genes that encode proteins directly involved in viral RNA synthesis are shaded in light gray. PMO compounds were designed to target either genomic (–) RNA (↔) or antigenomic (+) RNA and mRNA (↔). Compounds binding to plus-stranded RNA were directed against the translation start site regions of either the VP35 gene (3136) or the L gene (11588) and the trailer region (18959). The 3' end of the genome is magnified to show the target locations of the three negative-strand sequences in detail: leader terminus (leader), transcription start signal of the NP gene (tss-NP), and a region promoting efficient replication (rep pro, 108). Nucleotide numbers refer to GenBank accession number AF086833. (B) Vero cells were pretreated with 5.0  $\mu$ M of the indicated compound for 3 h and subsequently infected with EBOV at an MOI of 5 TCID<sub>50</sub> per cell. After the inoculum was removed, DMEM with 2% FCS containing 5  $\mu$ M P-PMO was added to the cells. Oligomer sequences and their target sites are listed in Table 1. At 48 hpi, supernatant was used to infect Vero cells in 96-well plates for a TCID<sub>50</sub> assay. On day 12 postinfection, the CPE was evaluated and the TCID<sub>50</sub>/ml calculated. The experiment was performed twice with similar outcomes.

**Mice.** C57Bl/6 mice were obtained from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD). The mice used in these studies were females aged 8 to 10 weeks at the start of these experiments. Mice were housed in a BSL-4 laboratory at USAMRIID in microisolator cages and provided water and chow ad libitum. Mice were treated with a 500- $\mu$ g dose of the indicated PMO compound at both 24 and 4 h before or 24 h after intraperitoneal injection with  $\sim$ 1,000 PFU, or  $\sim$ 30,000 99% lethal dose, of mouse-adapted EBOV (4) diluted in PBS. After challenge, animals were observed for illness at least twice daily for at least 28 days. Some mice were weighed once daily as a measure of illness (4). Blood samples ( $n = 6$  mice per group) were obtained on 3 and 6 dpi under anesthesia by cardiac puncture. Viral titers in the serum were determined by traditional plaque assay (25), and IFN- $\alpha$  levels were determined using a commercially available enzyme-linked immunosorbent assay per the manufacturer's directions under BSL-4 conditions (PBL Laboratories, Piscataway, NJ).

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the *Guide for the Care and Use of Laboratory Animals* (28a). The facility where this research was conducted (USAMRIID) is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

**Statistical analysis.** A paired Student *t* test was used to directly compare treated and mock-treated samples (viral titers and cytokine levels). The proportions of VP35-specific P-PMO-treated and scramble P-PMO- or PBS-treated mice were compared by one-tailed Fisher exact tests within experiments. A *P* value of  $\leq 0.05$  was considered significant.

## RESULTS

**A VP35-specific P-PMO inhibits Ebola virus replication in cell culture.** To facilitate cellular uptake, all PMO used in the cell culture studies were covalently conjugated to an arginine-rich peptide. For our initial studies, antisense P-PMO were designed against various sequences in the EBOV genome or antigenome and mRNAs (Table 1 and Fig. 1A). Three of the compounds were complementary to negative-sense RNA and targeted sequences within the genomic replication promoter (leader, 108) (41) and the transcription start signal of the first gene (tss-NP). The other three antisense P-PMO were directed

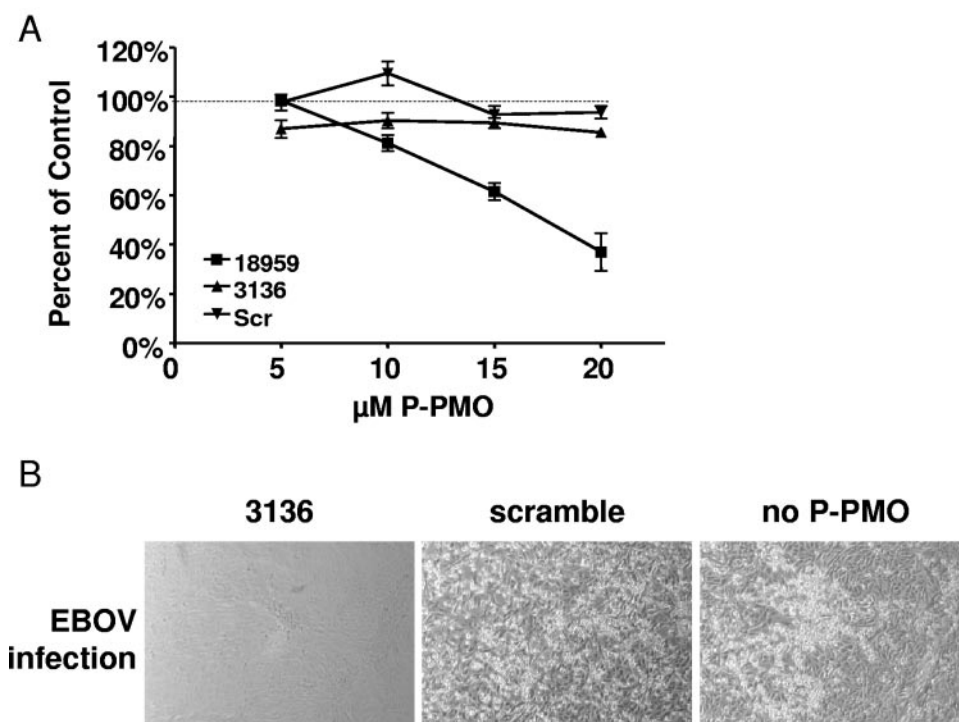


FIG. 2. Evaluation of cytotoxicity and efficacy of EBOV-specific P-PMO compounds. (A) Vero cells were incubated with 0 to 20  $\mu\text{M}$  of the various P-PMO. After 24 h, the cells were examined for viability using an MTT assay. The data are plotted as percent changes in viability in comparison to untreated control Vero cells. Standard deviations of the means of triplicate samples are indicated by bars. (B) Light microscopy was employed to observe the CPE caused by either EBOV infection or cytotoxicity of the oligomers. Vero cells were preincubated with 5.0  $\mu\text{M}$  of 3136 or scramble P-PMO for 3 h. Cells were then infected with EBOV at an MOI of 5 TCID<sub>50</sub> per cell. The inoculum was removed, fresh DMEM was supplemented with 2% FCS, and the appropriate P-PMO at 5.0  $\mu\text{M}$  was added. Cells were checked daily for CPE and photographed under light microscopy. Pictures shown here were taken at 6 dpi.

against positive-sense RNA: the translation start site regions of the VP35 (3136) and the L genes (11588) and a sequence within the antigenomic replication promoter (18959). As a control for nonspecific effects, we used a nonsense sequence (scramble) that had no significant sequence identity with EBOV or known human or nonhuman primate mRNA sequences. To determine the relative antiviral efficacies of the various P-PMO, Vero cells were pretreated for 3 h with 5  $\mu\text{M}$  of P-PMO and then infected with EBOV with an MOI of 5 TCID<sub>50</sub> per cell. As seen in Fig. 1B, the 3136 and 18959 P-PMO targeting the VP35 gene and trailer, respectively, each reduced viral titers approximately 100-fold at 48 hpi. The tss-NP and 11588 P-PMO reduced viral titers about 10-fold, while the 108 P-PMO, targeting the genomic replication promoter region, and scramble P-PMO did not appear to affect the level of EBOV replication compared to the untreated group.

MTT cytotoxicity test and CPE analysis on P-PMO-treated cells were performed under cell culture conditions as described above. The goal of these studies was to determine the cellular cytotoxicity and the antiviral effects of the 18959 and 3136 P-PMO. MTT analysis revealed that at concentrations over 10  $\mu\text{M}$ , 18959 P-PMO was increasingly cytotoxic; however, little cytotoxicity was observed in cells treated with up to 20  $\mu\text{M}$  3136 P-PMO (Fig. 2A). When cells were infected with EBOV and analyzed by light microscopy, morphological changes indicative of infection and CPE were clearly visible at 3 to 4 dpi

and severe at 6 dpi in the untreated or scramble P-PMO-treated cells (Fig. 2B and data not shown). Infected cells that were treated with 3136 P-PMO did not show any evidence of CPE for up to 6 dpi. CPE started to occur at 7 dpi in infected cells treated with 3136 P-PMO (data not shown). Because of concern that much of the antiviral effects of 18959 P-PMO could be attributed to cytotoxicity, we decided to focus our efforts on investigating the properties of the VP35-specific 3136 P-PMO.

**Inhibition of EBOV replication by VP35-specific PMO compounds is dose, time, and sequence specific.** To further characterize the 3136 P-PMO inhibition of viral replication in cell culture, dose-response and time course experiments were carried out. Cells were pretreated with 3136 P-PMO or scramble P-PMO doses from 0.1 to 5  $\mu\text{M}$  and infected with an MOI of 5 TCID<sub>50</sub> per cell. At 48 hpi, supernatants were harvested for analysis by a TCID<sub>50</sub> assay. In parallel, total cellular RNA was extracted and analyzed in a semiquantitative RT-PCR assay for genomic EBOV RNA (with primers designed to amplify a fragment of VP35/VP40 genes) and GAPDH mRNA as an internal control. PCR products were quantified by densitometry from electrophoresed gels. Based on the TCID<sub>50</sub> analysis, the EC<sub>50</sub> was  $0.9 \pm 0.1$   $\mu\text{M}$  for 3136 P-PMO and  $3.3 \pm 0.3$   $\mu\text{M}$  for scramble P-PMO. The RT-PCR assay yielded similar results, with an EC<sub>50</sub> of  $1.25 \pm 0.2$   $\mu\text{M}$  for 3136 P-PMO and  $4.3 \pm 0.3$   $\mu\text{M}$  for scramble P-PMO. We next compared the inhibitory effects of 3136 and scramble P-PMO on cells infected with an



MOI of 5 or 0.05. The 3136 P-PMO reduced viral titers nearly 100-fold, independent of the amount of input virus (Fig. 3A, left panel). Application of the scramble P-PMO reduced the viral titers 10-fold, also independent of the MOI (Fig. 3A, left panel). Similar results were obtained using RT-PCR to evaluate P-PMO-treated cells infected with an MOI of 5. Application of the scramble P-PMO resulted in a decrease of viral titers by about 35%, while the 3136 P-PMO resulted in a decrease of ~95%, compared to infected cells without P-PMO (Fig. 3A, right panel). Together, these results suggest that RT-PCR of viral RNA could be used as an alternative method to TCID<sub>50</sub> analysis to determine the effect of P-PMO on viral replication. However, the lowest detectable titers using RT-PCR were ~10<sup>4</sup> TCID<sub>50</sub>/ml. Since infection with an MOI of 0.05 yielded viral titers of less than 10<sup>4</sup> TCID<sub>50</sub>/ml (Fig. 3A), viral RNA could not be detected by RT-PCR in these samples. We suspect that the low sensitivity of the RT-PCR assay could have been caused by the length of the amplicon (1,119 nucleotides).

To examine how the timing of P-PMO treatment affected EBOV replication, treatment of Vero E6 cells with P-PMO was initiated 3 h before, at the time of (0 h), or 4 h, 8 h, or 24 h after infection (MOI = 5). Cells treated with 3136 P-PMO starting at 3 h before EBOV inoculation yielded virtually no EBOV-specific RT-PCR product at 48 hpi. When treatment began at 0, 4, or 8 hpi, the levels of viral RNA were low but detectable, and much less inhibition was apparent when P-PMO was added at 24 hpi (Fig. 3B). An application of scramble P-PMO starting at 3 h preinoculation reduced viral replication only slightly (Fig. 3B). Although it would be unexpected due to the lack of sequence homology, we tested whether the 3136 P-PMO could affect MARV infection. As expected, neither the EBOV-specific 3136 nor scramble P-PMO affected MARV RNA levels (Fig. 3C). The band intensities generated by the RNA input quantity control (GAPDH mRNA) for all of the samples were nearly identical (Fig. 3B and C; data not shown).

To directly examine the effect of PMO and P-PMO on translation of EBOV sequence RNA, a cell-free *in vitro* translation system was used. In the interest of developing an optimal antiviral compound, the efficacy of the 22-mer VP35-specific 3136 P-PMO was compared to that of 3136 PMO varying in length from 16 to 22 base residues. A reporter plasmid was constructed with a sequence corresponding to the 5' portion of the VP35 mRNA (bases -98 to +39; in relation to the AUG start site) fused to the coding sequence of firefly luciferase. Various concentrations of the PMO compounds were added to reaction mixtures containing EBOV-luciferase RNA and rabbit reticulocyte lysate, and the light emissions were measured. High levels of light emission were observed in untreated *in vitro* translation reactions or when a scramble PMO compound was included in the reaction (Fig. 4). In contrast, the 22-residue VP35-specific 3136 PMO and P-PMO strongly inhibited light emission (Fig. 4). The EC<sub>50</sub> for 22-mer 3136 PMO was approximately 1 μM, and the corresponding 3136 P-PMO was approximately 0.07 μM. The shorter 16- and 19-residue 3136 PMO generated only minor inhibition of the reporter signal compared to untreated control samples, even at the highest concentration tested (30 μM). VP35-specific 3136 P-PMO of various lengths were not available for potency test-

ing; however, Nelson et al. reported observing that increasing P-PMO length resulted in increasing potency using *in vitro* translation assays similar to those described above (29).

**Protection of mice from lethal EBOV challenge.** To determine the ability of nonconjugated VP35 PMO to offer *in vivo* protection from lethal EBOV infection, C57Bl/6 mice were pretreated by intraperitoneal injection at 24 and 4 h with 500 μg per dose of 3136 PMO, leader PMO, or scramble PMO. 3136 PMO greatly increased the survival of the EBOV-infected mice (Fig. 5A) compared to the mice treated with either leader ( $P < 10^{-4}$ ) or scramble ( $P < 10^{-7}$ ) PMO. As the length of VP35-targeted PMO affected their ability to inhibit translation in cell-free assays, we tested whether a similar phenomenon would be observed *in vivo*. As shown in Fig. 5B, 3136 PMO of 22, 19, or 16 base residues generated protection rates of 85%, 70%, and 50%, respectively ( $P < 10^{-3}$ , 0.01, and 0.07, compared to the scramble PMO or PBS-treated mice). Lastly, in order to gain insight into the behavior of P-PMO *in vivo*, we tested the effect of the peptide-conjugated 3136 P-PMO compared to that of the nonconjugated 3136 PMO (Fig. 5C). Survival of mice treated with 3136 P-PMO treatment was somewhat higher than that of mice treated with 3136 PMO (100% and 85%, respectively); however, this difference was not significantly different ( $P = 0.052$ ). As expected, neither the scramble PMO nor P-PMO provided protection from lethal EBOV infection (Fig. 5C).

While a prophylactic treatment for EBOV infections may well be useful for medical and laboratory personnel, a postinfection therapeutic is, of course, highly desirable. Therefore, we tested whether a single dose of the VP35-specific P-PMO could enhance survival when administered 24 h after lethal EBOV challenge. Mice treated intraperitoneally with the 3136 P-PMO were completely protected from lethal EBOV infection, whereas mice similarly treated with the scramble P-PMO died after infection ( $P < 10^{-7}$ ) (Fig. 6A). In addition, the VP35-specific P-PMO-treated mice showed no clinical signs of illness through 28 days of observation postchallenge. As an indicator of oligomer toxicity and/or EBOV-induced illness, we also weighed the mice daily during the infection study (Fig. 6B). While the 3136 P-PMO-treated mice lost less than 5% body weight over the duration of the study, the scramble control P-PMO-treated mice showed weight loss starting at 4 days postchallenge, consistent with the weight loss observed in naive mice following EBOV infection (data not shown) (4). Further, none of the surviving mice in any of the treatment groups in this study appeared, by cageside observation, to suffer adverse effects from PMO or P-PMO treatment. Compared to naive mice, the scramble control P-PMO-treated mice did not exhibit an increase in time to death or in their survival rate (Fig. 5 and 6A; data not shown). Examination of the viral titers in the blood serum of P-PMO-treated mice showed that viral titers were reduced in the VP35-specific P-PMO-treated mice compared to those of the scramble P-PMO-treated mice (Fig. 6C). This difference was statistically significant only at 6 days postchallenge ( $P = 0.039$ ). We also determined whether the P-PMO treatment caused any modulation in IFN levels. This was of particular interest, as the VP35 gene product has been shown to be an IFN antagonist and the EBOV mouse model is highly sensitive to IFN induction (1, 2, 4). However, there were no significant differences in IFN-α levels between the scramble and VP35-specific P-PMO-treated mice (Fig. 6D). Together,

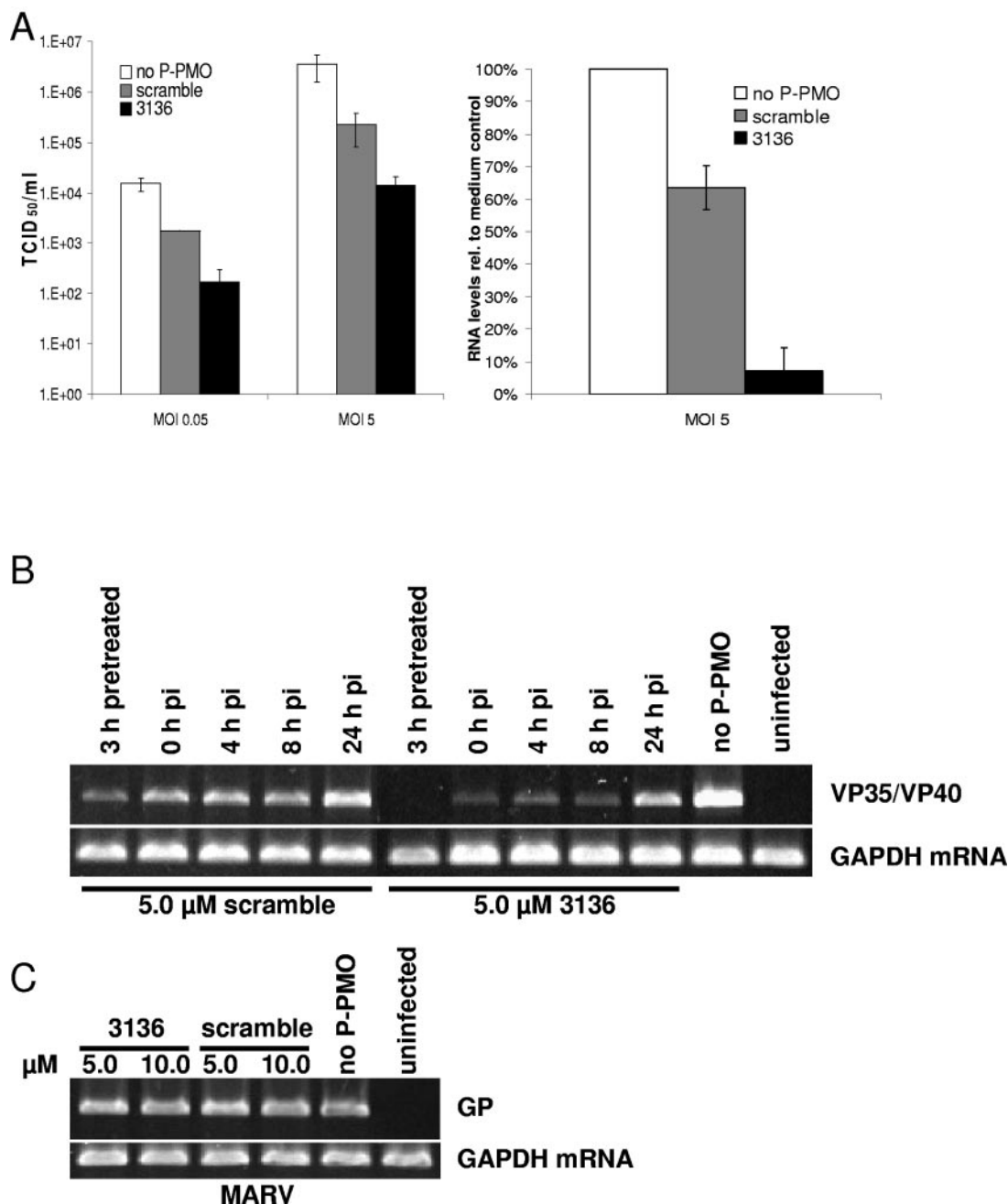


FIG. 3. Inhibition of EBOV replication by the VP35-specific P-PMO is dose, time, and sequence dependent. (A) Vero cells were pretreated with 5  $\mu$ M of the 3136 or scramble P-PMO for 3 h prior to infection with EBOV (MOI of 5 and 0.05 TCID<sub>50</sub> per cell, respectively). After 1 h, the inoculum was removed and replaced with DMEM containing 2% FCS and P-PMO at 5  $\mu$ M. At 48 hpi, supernatants from the P-PMO-treated and EBOV-infected cells were used to inoculate Vero cells in 96-well plates for a TCID<sub>50</sub> assay. The CPE remained constant after 11 dpi, and the TCID<sub>50</sub>/ml was calculated (left panel). The cells were lysed, and total RNA was isolated and used as a template for RT-PCR, with primers designed to amplify 1,119 nucleotides of EBOV genomic RNA comprising sequence from the 5' region of VP35 gene and 3' region of VP40 gene, or GAPDH mRNA as a control. Products were separated by agarose gel electrophoresis and quantified after staining. The amount of EBOV-specific RNA was normalized with the GAPDH control, and reduction was calculated by setting EBOV-specific signals from infected cells without P-PMO treatment at 100% (right panel). Error bars represent the standard errors of the means of three replicates. (B) Compounds were added to Vero cells seeded in 24-well plates at the indicated time points: 3 h prior to infection, immediately after removal of inoculum (0 hpi), or at 4, 8, or 24 h after infection with EBOV (MOI = 5 TCID<sub>50</sub> per cell). Cells were harvested at 48 hpi, total RNA was isolated, RT-PCR was performed, and the products were resolved on a 2% agarose gel stained with ethidium bromide. (C) Vero cells were preincubated with 5 or 10  $\mu$ M of 3136 or scramble P-PMO. After 3 h, cells were infected with MARV at an MOI of 5 TCID<sub>50</sub> per cell. The inoculum was removed at 1 hpi and replaced with fresh DMEM containing 2% FCS and the respective P-PMO. At 24 hpi, cells were harvested and total RNA was isolated. RT-PCRs (of a segment of GP) from genomic viral RNA template and cellular GAPDH mRNA, as a control, were performed. The experiments shown in this figure are representative of three experiments of similar design and outcome.



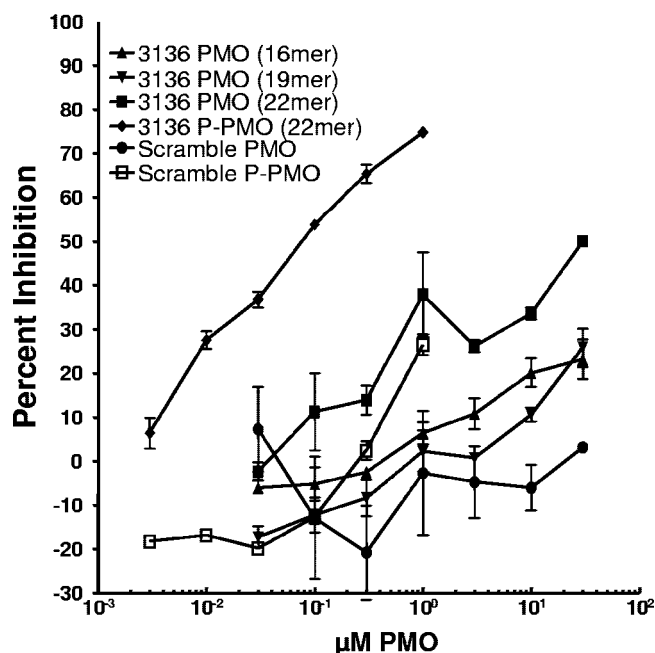


FIG. 4. Sequence-specific inhibition with VP35 PMO compounds in cell-free translation assay. RNA representing the 5' 137 nucleotides of VP35 mRNA sequence, which includes the 3136 target site followed by the coding sequence for firefly luciferase, was in vitro transcribed from a reporter plasmid. Various concentrations of oligomers were added to rabbit reticulocyte lysate in vitro translation reactions in the presence of 1 nM RNA (see figure inset for oligomers tested). The graphed line represents the mean light units of three treatment wells per data point. Data are represented as the mean percent inhibitions of reporter signals of triplicate treatment wells in comparison to the means of water-treated control reactions.

our in vitro and in vivo studies suggest that the VP35-specific PMO and P-PMO generate protection by reducing virus amplification.

## DISCUSSION

In this study we tested six EBOV-specific P-PMO in cell culture and identified a single agent that efficiently inhibited Zaire EBOV amplification in a nontoxic manner. The 22-mer P-PMO targeting EBOV VP35 inhibited EBOV amplification in cell culture in a time-, sequence-, and dose-dependent manner and was also capable of providing effective prophylactic or postinfection treatment to EBOV-infected mice. The nonconjugated form of the 22-mer VP35-specific PMO also efficiently protected mice prophylactically from lethal EBOV infection. Although postinfection treatment with nonconjugated PMO was not attempted in this study, it has also been reported as highly effective in this EBOV mouse model (38a).

With the use of minigenome and infectious clone systems, a critical role for VP35 in the EBOV and MARV replication and transcription complexes has previously been demonstrated (38). Recently, an alternate antiviral approach employing transfection of small interfering RNAs against NP, VP30, and VP35 before MARV infection resulted in decreased production of viral proteins (9). The most plausible mechanism of action of the VP35-directed PMO compounds is the formation of a

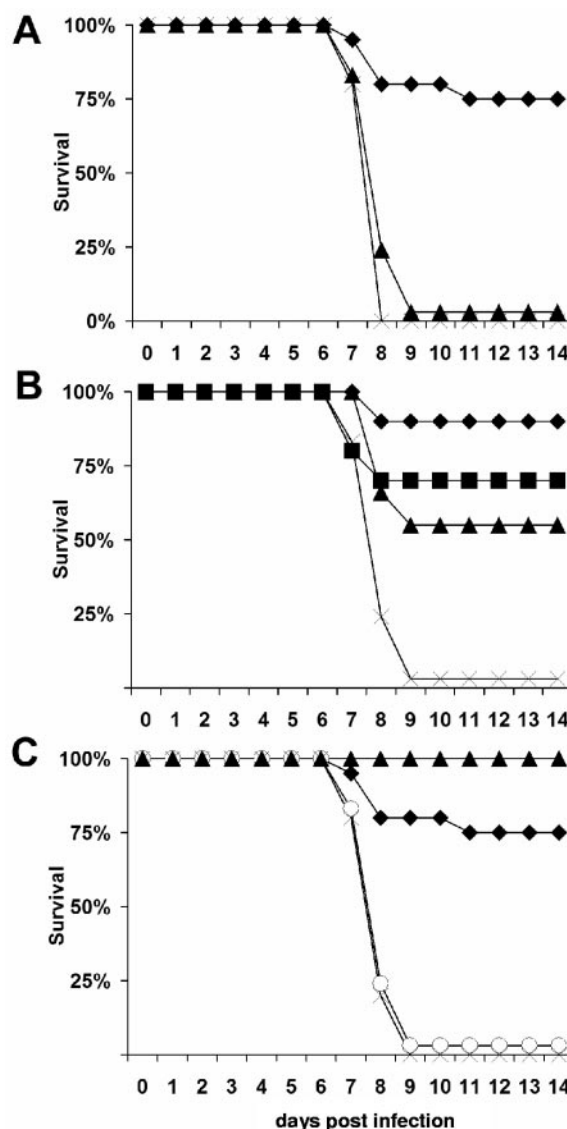


FIG. 5. Prophylactic treatment with antisense PMO compounds targeting VP35 increases survival of mice infected with EBOV. (A) Kaplan-Meier survival curve showing mice pretreated via intraperitoneal injection at 24 and 4 h before EBOV infection with 500-μg doses of 3136 22-mer (◆), leader (▲), or scramble (×) PMO. The mice were challenged with ~1,000 PFU of mouse-adapted EBOV, and the results are plotted as percent survival for each group ( $n = 20$  to 30 per group). (B) Survival of mice treated intraperitoneally with 500-μg doses of 3136 22-mer (◆), 19-mer (■), 16-mer (▲), or scramble (×) PMO at 24 and 4 h before infection with ~1,000 PFU of mouse-adapted EBOV. The data are plotted as percent survival for each group ( $n = 10$  to 20 per group). (C) Survival curves of mice treated 24 and 4 h via intraperitoneal injection before EBOV infection with 500-μg doses of 3136 22-mer PMO (◆), 3136 22-mer P-PMO (▲), scramble PMO (×), or scramble P-PMO (○). The data are presented as Kaplan-Meier survival curves ( $n = 10$  to 20 per group). All mice were observed for at least 28 dpi, and no changes in survival were noted from 14 to 28 dpi.

duplex with VP35 mRNA and subsequent interference with the assembly of the translation initiation complex at the initiator AUG region, thereby reducing or preventing translation of VP35 protein (12). As the VP35 oligomers target the se-

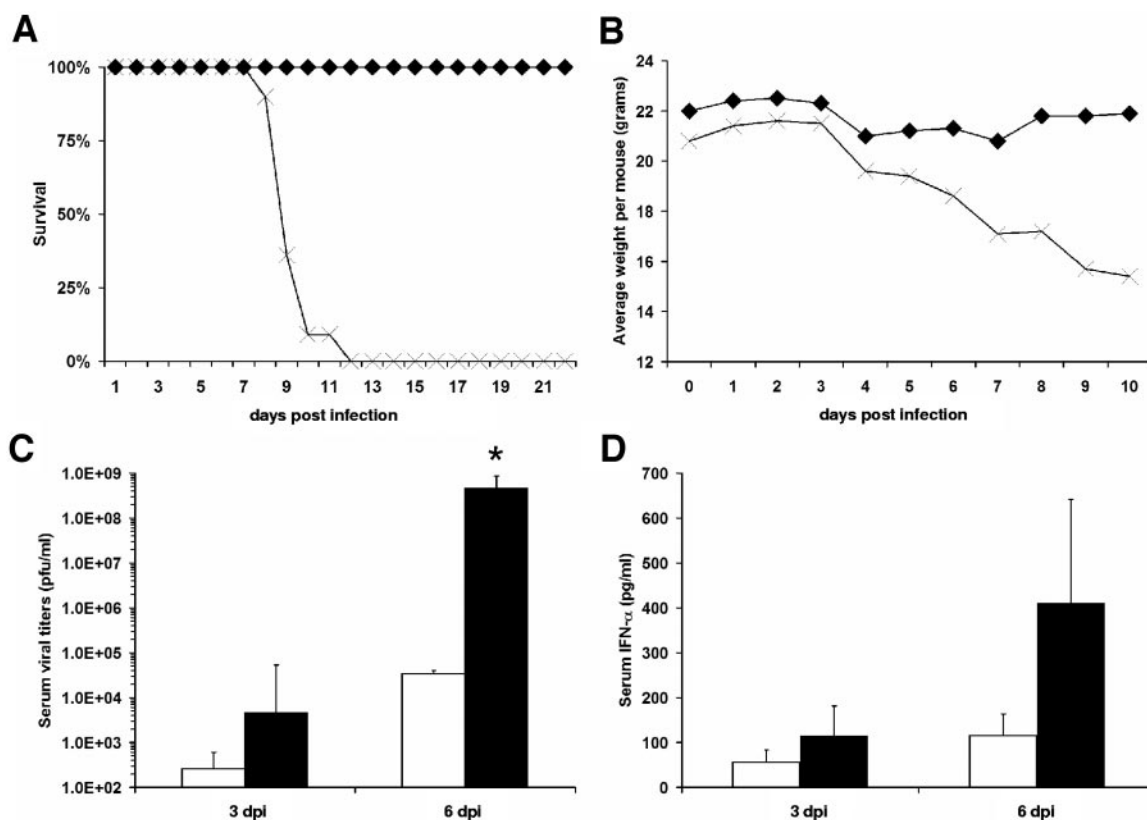


FIG. 6. Prevention of lethal outcome in EBOV-infected mice treated therapeutically with VP35-specific P-PMO. (A) Kaplan-Meier survival curve showing mice treated intraperitoneally with a single 500- $\mu$ g dose of VP35-specific 3136 22-mer P-PMO ( $\blacklozenge$ ) or the scramble P-PMO ( $\times$ ) at 24 h after receiving  $\sim 1,000$  PFU of mouse-adapted EBOV infection. The results are plotted as percent survival for each group ( $n = 12$  per group). All mice were observed for at least 28 dpi, and no changes in survival were noted from 21 to 28 dpi. (B) The 3136 ( $\blacklozenge$ ) or scramble ( $\times$ ) P-PMO mice were weighed daily following lethal EBOV infection. Data are representative of the mean weight of the surviving mice in each group, which were weighed daily en masse ( $n = 12$  per group). (C and D) Blood samples from the 3136 (white bars) or scramble (black bars) P-PMO mice were obtained on day 3 and day 6 under anesthesia by cardiac puncture. Viral titers in the serum were determined by plaque assay (C), and IFN- $\alpha$  levels were determined using a commercially available enzyme-linked immunosorbent assay (D). Data are shown as means ( $n = 6$  mice per group) and standard deviations (viral titers) or standard errors of the means (IFN). Significant differences ( $P < 0.05$ ) between the VP35-specific 3136 and scramble P-PMO-treated group are indicated by an asterisk.

quence found in the full-length antigenomic replicative intermediate, as well as in the VP35 mRNA, we cannot rule out interference in viral genomic RNA synthesis as possibly contributing to the observed reduction in EBOV amplification. It is currently unclear whether interference with the interferon antagonist function of VP35 (1) contributed to in vivo efficacy of the 3136 oligomers; however, our current data do not support this notion as there were no significant differences in IFN- $\alpha$  levels between EBOV-infected mice treated with the 3136 or scramble P-PMO (Fig. 6D). To date, in vitro demonstrations of the interferon antagonistic properties of VP35 have not been repeated using in vivo animal models. Of note, EBOV infection of nonhuman primates and mice results in high levels of serum IFN- $\alpha$  (Fig. 6D) (16, 23). Future studies will include the utilization of PMO compounds as tools to investigate the functional role of VP35 and other viral proteins and RNA elements in the filovirus life cycle.

All of the viral sequences targeted by the P-PMO in these studies are located in domains thought to be critical for efficient viral amplification, yet only the VP35 (3136) and trailer-targeted (18959) oligomers showed efficacy. Although 18959

showed high efficacy at 5  $\mu$ M, the inexplicable cytotoxicity exhibited at 20  $\mu$ M lessened our enthusiasm for further testing of this compound. Why the four other antisense P-PMO were ineffective against EBOV in this cell culture model is unclear at this time. Such negative results leave the question of whether an oligomer was unable to base pair effectively to its intended target or whether base pairing did occur but did not result in significant interference with any critical viral molecular events. We suspect that the three compounds that were designed to target EBOV genomic RNA may have been hindered or unable to duplex with their target sequences due to the physical inaccessibility of negative-strand viral RNA. The tight encapsidation of filovirus genomic RNA has been demonstrated by stability of the nucleocapsid in high salt concentrations (3) and its resistance to micrococcal nuclease activity after treatment with nonionic detergents (27, 28).

In the current studies, we have used both peptide-conjugated and nonconjugated PMO. The  $R_0F_2$  peptide used here enhances uptake of PMO by cells in culture (26, 31). Furthermore, P-PMO have been shown to generate a higher degree of antisense efficacy than corresponding nonconjugated PMO of

the same base sequence, independent of delivery considerations (29), and this phenomenon was evident here as well in the results of the cell-free translation comparison between the 3136 PMO and 3136 P-PMO 22-mers. However, at the dose employed in this study (0.5 mg), the peptide conjugate does not appear to be critical in achieving a high degree of in vivo protection against lethal EBOV infection in this mouse model, and there was not a large difference in survival between mice treated with the conjugated or nonconjugated 22-mer 3136 compounds (Fig. 5C). Not surprisingly, an incremental increase in length (from 16 to 22 base residues) of VP35-specific PMO generated corresponding incremental increases in cell-free and in vivo efficacy.

This report is one of the first demonstrations of in vivo protection against a member of the *Mononegavirales* by using antisense-mediated viral gene expression knockdown. Previously, Leaman et al. showed that a modified phosphorothioate antisense oligomer designed to target repetitive gene start sequences within the respiratory syncytial virus genome could reduce viral titers in respiratory syncytial virus-infected monkeys up to 10,000-fold (22). Our current studies suggest that the VP35-directed PMO and/or P-PMO hold potential as therapeutics for EBOV infection and merit consideration for efficacy testing in a nonhuman primate model.

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